Project Report

STUDY OF EFFECTS OF PYRIPROXYFEN, A JUVENILE HORMONE ANALOGUE ON THE DEVELOPMENT OF *DROSOPHILA MELANOGASTER*

Dissertation submitted to the University of Kerala in partial fulfillment of the requirements for the award of the degree of

Bachelor of Science
in
ZOOLOGY

(B.Sc Zoology, 2014- 17 batch)

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DEPARTMENT OF ZOOLOGY

T K M COLLEGE OF ARTS AND SCIENCE

KOLLAM-5

March-2017

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CERTIFICATE

This is to certify that the dissertation entitled **Study of Effects of Pyriproxyfen, a Juvenile Hormone Analogue on the Development of *Drosophila melanogaster*** is an authentic record of the work done by.....with Reg. No:..... under my supervision as partial fulfillment of the requirements for the Degree of *Bachelor of Science* in Zoology and this report has not been submitted earlier for the award of any degree or diploma or any other similar titles anywhere.

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Certified bona fide:

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EXAMINERS:

1.

2.

DECLARATION

I do hereby declare that this dissertation entitled **Study of Effects of Pyriproxyfen, a Juvenile Hormone Analogue on the Development of *Drosophila melanogaster*** is a bonafide report of the project work carried out by me, under the supervision and guidance of Aseeb A K, Asst. Professor, Department of Zoology, TKM College of Arts and Science, Kollam as partial fulfillment of the requirements for the award of the Degree of Bachelor of Science in Zoology.

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27.03.2017

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students

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DEDICATED TO MY PARENTS AND TEACHERS....

INTRODUCTION

1 INTRODUCTION

Drosophila melanogaster is a species of small flies, belonging to the genus *Drosophila* and family Drosophilidae. The entire genus of *Drosophila* contains more than 1500 species and is very diverse in appearance, behavior and breeding habit. They are found all around the world, with more species in the tropical regions. They can be found in deserts, tropical rain forest, cities, swamps and alpine zones. Most species breed in various kinds of decaying plant and fungal material, including fruit, bark, slime fluxes, flowers and mushrooms. *Drosophila melanogaster* is a fruit fly, a little insect about 3mm long, of the kind that accumulates around spoiled fruit. It is a common pest in homes, restaurants, and other occupied places. It is also one of the most valuable of organisms in biological research, particularly in genetics and developmental biology. *Drosophila melanogaster* has been used as a model organism for research for almost a century, and today, several thousand scientists are working on many different aspects of the fruit fly. *D. melanogaster* exhibits sexual dimorphism: females are about 2.5mm long; males are slightly smaller with darker backs. Males are easily distinguished from females based on colour differences, with a distinct black patch at the abdomen, less noticeable in recently emerged flies and the sex combs (a row

of dark bristles on the tarsus of the first leg) Further more males have a cluster of spiky hairs (claspers) surrounding the reproducing parts used to attach to the female during mating. *D. melanogaster* is closely associated with humans and are often referred to as domestic species.

1.1 Life History of *Drosophila melanogaster*

Drosophila melanogaster display a holometabulous method of development, meaning that they have 4 distinct stages of their life cycle, each with a radically different body plan: egg, larva, pupa and finally adult. The eggs have one or more respiratory filaments near the anterior end; the tips of these extend above the surface and allow oxygen to reach the embryo. Larvae feed not on vegetable matter itself but on the yeasts and micro organisms present on the decaying breeding substrate. Development time varies widely between species and depends on the environmental factors such as temperature, breeding substrates and crowding.

Fruit flies begin their lives as an embryo in an egg. This stage lasts for about one day. During this time embryo develops into a larva. The larva is white, segmented and worm like. The larval stage is a feeding stage and consists of three subdivisions called instars.

The first instar larva hatches out of the egg, crawls into a food source and eats. The larva in each stage eats as much as possible. After a day, the first instar larva molts and becomes the second instar larva. Again the larva in

this stage eats and eats. After a day in this stage, the larva molts again to become the third instar larva. After 2 days of eating in this stage, the larva crawls out of the food source and molts again.

Following this molt, the larva stops moving and forms a pupa. *Drosophila* stays in the pupa for about five days. During this time, the metamorphosis or change from larva to adult is occurring. Adult structures like, wings, legs and eye develop.

When the adult emerge from pupa, they are fully formed. They become fertile after about 10 hours, copulate, the female lay eggs, and the cycle begins again. The whole life cycle takes about 10 -12 days.

The first, second and third instars of *Drosophila* can distinguished only by their size differences. Second instar larvae are twice the length of first instar. Third instar larvae is twice the length of second instar.

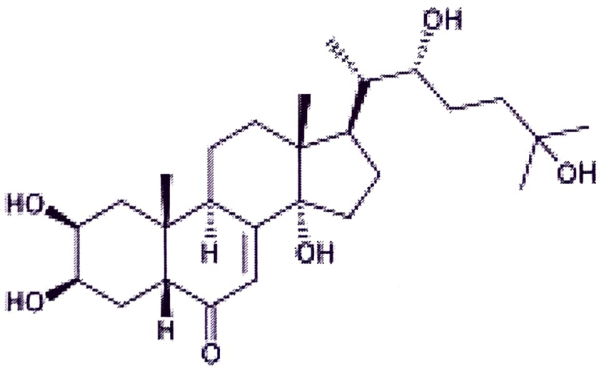
Adult *Drosophila* is small flies, typically pale yellow to reddish brown to black, with red eyes. The plumose arista, bristling of the head and thorax, and wing venation are characters used to diagnose the family. Most are small about 2-4 millimeters long.

1.2 Insect Hormones of *Drosophila melanogaster*

Almost every aspect of an insect's life is regulated by hormones at one time or another. Molting and metamorphosis are the most obvious of the

endocrine stimulated events in the insect life cycle, and the best studied. But hormones also control such disparate physiological and developmental phenomena as metabolism, water balance, seasonal polymorphisms, caste determination, reproductive cycles and diapause as well as behaviors such as eclosion, pheromone production, migration and social dominance. Insect hormones have a pervasive role in the regulation of post embryonic development.

1.2.1 Ecdysone



Ecdysone is a steroidal prohormone of the major insect molting hormone 20-hydroxy ecdysone, which is secreted from the prothoracic glands. Insect molting hormones (ecdysone and its homologues) are generally called ecdysteroids. Ecdysteroids act as molting hormones of arthropods but also occur in other related phyla where they can play different roles.

In *Drosophila melanogaster*, an increase in ecdysone concentration induces the expression of genes coding for proteins that the larva requires and

it cause chromosome puffs (sites of high expression). In them 20- hydroxyl ecdysone enhances the expression of some of the antibacterial peptides such as dipteran that are involved in the suppression of bacterial infection and juvenile hormone suppresses this enhancement (Flatt *et al.*, 2008).

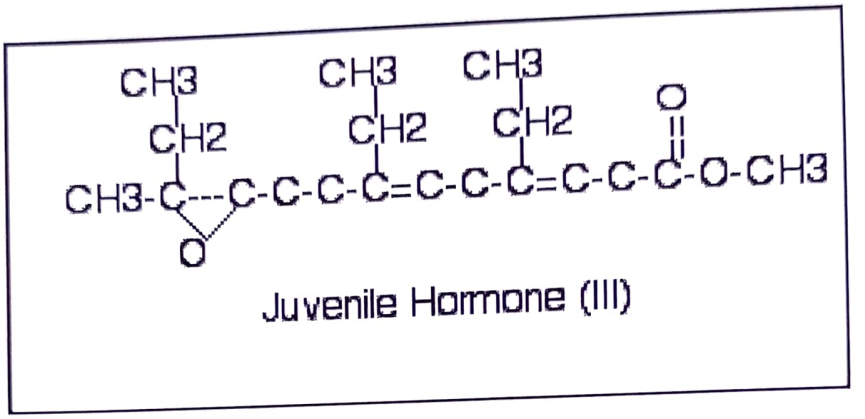
Ecdysteroids also appear in many plants mostly as a protection agent (toxins or antifeedants) against herbivorous insects. Ponasterone A was the first isolated phytoecdysteroid (Nakanishi *et al.*, 1966). Since then many synthetic and natural steroids with molting hormone activity have been discovered. These include four groups: phytoecdysoids, zooecdysoids, synthetic ecdysoids and non steroidal ecdysone agonist.

1.2.2 Juvenile Hormone

Juvenile hormone was discovered in 1965 and the molecular structure solved in 1967. Juvenile hormones are secreted by a pair of endocrine glands behind the brain called the corpora allata. They are a group of acyclic sesquiterpenoids that regulate many aspects of insect physiology. It has a wide range of functions in regulating development and physiological process such as metamorphosis, caste determination, ovarian maturation, diapause and migration in insects (Riddiford., 1994 1996, Wyatt & Davy., 1996, Goodman and Granger., 2005).

Most species contain only Juvenile hormone JH III. To date JHO, JHI, JH11 have been identified only in the Lepidoptera. The form JHB3 (JH III

bisepoxide) appears to be the most important JH in Diptera. JH III was identified in whole body extracts of eggs, larvae, pupae, pharate adults and adults of *Drosophila melanogaster*.



Juvenile Hormone (III)

1.2.3 Effects of Juvenile Hormone in Insects

A holometabolous insect molt several times during the larval stages and then undergoes metamorphosis, first into a pupa and then into an adult. These processes are largely regulated by JH and the steroid hormone ecdysone (Riddiford *et al.*, 2001). In the presence of JH, ecdysone causes a molt to a similar stage, but in the absence of JH, ecdysone causes a metamorphic molt (Riddiford., 1994). This effect of JH is defined as its "status quo" action (Williams ., 1952).

1.3 Insect Growth Regulators (IGRs)

Following world war II, the synthetic organic insecticides quickly achieved wide acceptance in the farming industry to that many agriculturists in various parts of the world started using there chemicals to suppress key

insect pests of major crops. But it has increasingly become evident that indiscriminate use of these insecticides will result not only in the development of resistant strains but also in the destruction of natural enemies of pest population. The awareness of these factors led to a demand for alternative chemicals for insect control.

IGRs are one of the fastest developing chemical classes of insecticides in the past ten years. The term Insect Growth Regulator was designed to describe a new class of bio rational compounds. Through greater selectivity of action these compounds appear to fit the requirements for third generation pesticides, such as the absence of undesirable effects on man, wild life and the environment and compatibility with modern insect pest management principles.

The IGRs belong to a class of compounds which interfere with normal growth, development and reproduction of insects. Mainly there are 3 categories of IGRs: (a) compounds which directly or indirectly influence the hormones regulating development and reproduction. (E g: Juvenile hormone analogues, antijuvenile hormone agents, ecdysone mimics, antiecdysteroids and neurohormone analogues and their antagonists) (b) Compounds which inhibit cuticle formation through an effect on chitin synthesis (e.g. Benzophenyl-ureas) and (c) compounds with miscellaneous mode of action (e g: Azadirachtin). These compounds are thought to be particularly attractive in

pest control programmes as they are ecologically stable and environmentally safer substances, to which insects are unable to develop resistance.

Research on insect hormones as biological discipline has developed extensively during the past forty years. There are three major categories of insect hormones: neurohormones secreted by the neurosecretory cells of brain and segmented ganglia, juvenile hormone (JH) secreted by Corpora allata and ecdystreoids secreted by the prothoracic glands. Although the genesis of the studies on Insect Endocrinology was purely academic, increasing attention has been focused in recent years on the potential for developing use of insect hormones as candidate insecticides. The enthusiasm for developing hormonal methods of control was based on the understanding that hormones regulate most of the critical physiological processes of insects like morphogenesis, reproduction and behavior. The integrated pest management(IPM) programmes exploit insects depending on hormones for a variety of their life activities. Elimination of hormones from the haemolymph or the presence of high titre of hormones at a time of low endogenous titre will cause a rearrangement of hormone dependent processes of morphogenesis and reproduction. Based on this concept certain hormone analogues (e.g. Analogues of JH and ecdysteroids) and antihormones (like anti JH agents, anti ecdysteroids and antineuropeptides) have already been developed. This approach is of considerable interest in IPM programmes because these

compounds are nonpersistent, biodegradable and more selective to insect pest species than the conventional insecticides.

1.4 Juvenile Hormone Analogues

Based on the studies of various physiological effects of JHs in insects, Williams (1967) suggested that this hormone or its analogues could be used as specific control agents to which pest species may be unable to develop resistance. This led to the discovery of juvenile hormone analogues (JHAs). The early juvenoid IGRs were true analogues of juvenile hormone and were unstable when exposed to U.V. light. This seriously limited their use in plant protection. Another group of juvenoid IGRs, called juvenile mimics or Juvenile Analogues was then discovered. Entomologist found that extracts of many plant tissues have juvenilizing effects, but they have different chemical structures from JHs and are much more stable. Williams referred to this class of compounds as "third generation pesticides" to follow the "second generation" chlorinated hydro carbons such as DDT and the "first generation" inorganic pesticides such as lead arsenate.

Advantages of JHAs are they are species specific, less or zero toxicity to other animals, their fast penetrance through the insect cuticle and they get degraded to non toxic compounds in a short time period.

It is clear that by changing JH titre at certain periods during life history will adversely affect the metamorphosis and reproduction of insects. Moreover such an induced titre disturbances have a domino effect and disrupt other hormonal functions. Being a hormone; the regulation of secretion, transportation from the secretary site to the target site; degradation, excretion and feed back control are all important biochemical mechanisms which an analogue can very well interface. For e g: hydroprene administration stimulated JH synthesis at low doses and inhibited synthesis at higher doses (Tobe & Stay., 1979). Sometimes analogues may escape from JH esterase activity. Some of the commercial products of JHAs are available in the market like Altosid, Enstar and Insegar. These compounds have been successfully used for the control of insects that are pest in adult stages like floods water mosquitoes, manure breeding flies and certain green house pests.

However it soon becomes apparent that the JHAs are active only against last larval and early pupal stages of dipterans. Hence they are not ideal for use against species in which the early larval stages are responsible for the crop damage.

1.5 Pyriproxyfen

Pyriproxyfen is a pyridine based pesticide which is found to be effective against a variety of arthropoda. It was introduced to the US in 1996

to protect cotton crops against whitefly. It has also found use protecting other crops and can also be used as a treatment for cat fleas.

Pyriproxyfen is JH analogue preventing larvae from developing into adulthood and thus rendering them unable to reproduce. Nowadays Pyriproxyfen is effectively used as an Insect Growth Regulator (IGR) as a part of Integrated Pest Management system (IPM).

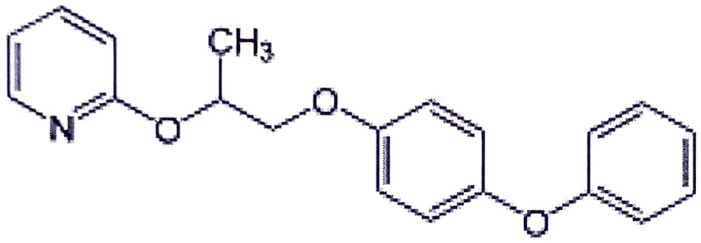


Fig. A. Structure of Pyriproxyfen

Name: 4 Phenoxy phenyl (RS)-2-(2-pyridy loxy) propyl ether 2-[1-(4-phenoxy phenoxy)] propan-2-yloxy] pysidine

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

2.1 *Drosophila melanogaster* Developmental Stages

Laboratory egg to adult development time between the relatively optimal temperatures of 20–27°C varied by about 1 week from the shortest reported time of 9.7 days to longest development time of 17.1 days. Currently, little is known about the effect of yeast on larval diet suitability, development time, and survival rate although work in other *Drosophila* indicates that yeasts increase host suitability (Tatum 1939; Becher et al. 2012). The importance of yeast as a protein source is known for *D. melanogaster* (Good and Tatar 2001; Tu and Tatar 2003), and appears necessary for egg production in lab-reared *Drosophila* (A. Wallingford, unpublished data).

A recent survey of life-history correlations in *Drosophila* reciprocally tested in different laboratories revealed substantial incongruence between test environments (Ackerman *et al.* 2001).

Chippindale *et al.* (1993) and Leroi *et al.* (1994 a,b) documented the apparent disappearance of the well-known life-history tradeoff between early fertility and late fertility/ survival described by Rose (1984). No general relationship exists between evolutionary rate and the time of genes expression. This may be true if the morphological conservation of

developmental time points depends on a small number of developmentally important genes (Arthur 1997; Raff 1996).

2.2 Role of Juvenile Hormone in Insect Development

A holometabolous insect molt several times during the larval stages and then undergoes metamorphosis, first into a pupa and then into an adult. These processes are largely regulated by JH and the steroid hormone ecdysone (Riddiford *et al.*, 2001). In the presence of JH, ecdysone causes a molt to a similar stage, but in the absence of JH, ecdysone causes a metamorphic molt (Riddiford., 1994). This effect of JH is defined as its "status quo" action (Williams., 1952).

Unlike lepidoptera and most other holometabolous insects, higher Diptera such as *Drosophila melanogaster* have lost most of their sensitivity to JH. Topical JH application cannot prevent the transition from larva to pupa in *D. melanogaster* (Ashburner.,1970; Postlethwait.,1974). Nevertheless application of high amount of JH or a JH mimic before or at the time of pupariation causes the formation of pupal–adult mosaic, a transparent abdomen covered with pupal cuticle with no or few short bristles and a relatively normal appearance of adult head and thorax (Ashburner.,1970; Postlethwait.,1974; Riddiford and Ashburner.,1991; Zhou & Riddiford., 2002).

Titers of the juvenile hormone III varied according to the stage of development. Highest levels were found in post feeding larvae and adults; only low levels were found in feeding last stadium larvae and in pupae. None of the other known JHs were detected (T.J. Sliter, B.J. Sedlak, F.C. Baker D.A. Schooley.,1987).

Using the hydroxyapatite assay, a JH III binding protein with a native molecular weight of nearly 400 kDa has been identified in the haemolymph of post feeding third instar larvae of *D. melanogaster*. The very high binding affinity and high specificity suggest that this JH III binding protein has a critical role in JH transport in the haemolymph (Lirim Shemshedini, Thomas, G. Wilson.,1988).

In all species, JH and their mimics, stimulate ovarian maturation and vitellogenin synthesis and that high doses of exogenous JHs or their mimics stimulate ovarian ecdysteroid synthesis at least in *Aedes aegypti, Aedes abropalpus,* and *D. melanogaster.* In *Musca domestica* and possibly *D. melanogaster*, 20-hydroxy ecdysone is present in the haemolymph at vitellogenic levels in newly emerged females and may persist in ovaryectomized adults. This may be the reason why topical application of JH or its mimics, to ovaryectomized isolated abdomens is effective in stimulating vitellogenin synthesis in the absence of the ovaries (Thomas J. Kely.,T.S. Adams, Margaret B. Schwartz, Mark T. Binnbaum, Elaine C. Rubenstein and

Richard B. Imberski.,1987). The production of control levels of vitellogenin requires both JH and 20- hydroxy ecdysone (T.S. Adams & P.A. Filipi.,1987).

Male last instar larvae and adult females of *Locusta migratoria* respond positively to the application of the juvenile hormone analogues methoperene and Pyriproxyfen, showing increase in vitellogenin gene expression (Dhadialla *et al.*,1987). Also in *Blatella germanica* females, vitellogenin gene expression is induced by topical application of JH (Comas *et al.*,1999). In Diptera it has been shown that the JH as well as ecdysone, are responsible for the induction of yolk protein gene expression (Hagedorn., 1994; Bownet.,1986, 1984).

Injection of *dl* juvenile hormone or C_{17} methyl ester into *Sarcophaga bullata* larvae prevents puparium formation or arrests development at about the 3rd day of pupal–adult development. Topical application to the abdomens of young pupae results in the secretion of a second pupal cuticle. This is the first reported morphogenetic effect of juvenile hormone on a fly (V.S. Srivastava and Lawrence I. Gilbert.,1968).

Application of Juvenoid on the Mediterranean fruit fly, *Ceratitis capitata* (Diptera, Tephritidae) within 24 hr before or after puparium formation caused disturbance in imaginal disc differentiation. Treated insects developed either into pupa-adult intermediates failing to emerge or into

defective adults whose fecundity was severely decreased (D.S. Daoud & F. Sehnal.,1974).

2.3 Application of Juvenile Hormone Analogue (JHA)

Already 500 analogues with JH activity has been discovered (Slama *et al.*,1974; Romanuk.,1981). Among the well known JHAs are, Epofenonane (Handgartner *et al.*,1976), Methoprene (Henrick *et al.*,1976). Hydroprene (Henrick *et al.*,1976) Kinoprene (Henrick *et al.*, 1976) and Phenoxyphenoxy carbamate (Peleg.,1982). The first JHA of commercial success were Methoprene and Hydroprene (C.A. Henrick, G.B staal and J.B. Siddal.,1973). Methoprene is active against dipteran insects and fleas and hydroprenes is active against cockroach. These compounds however, were too unstable under field conditions to be used for agriculture. Dorn *et al.* (1981) reported on the photostable JH analogue, fenoxycrab. Fenoxycrab was effective not only on household pest but also on agricultural pest such as leaf rollers, the codling moth and *psylla pyricola.*

Several JHAs were later synthesized and evaluated them for the control of household and agricultural pests, finally discovering pyriproxyfen as one of the most potent JHAs known to date (Masachika Hirano *et al.*,1998).

A few JHAs interfere with the activity of others hormones. Methoprene inhibits the secretion of prothoracicotropic hormone. This results in an inhibition in the secretion of ecdysone in *Mamestra brassicae* (Hiruma *et al.*,

1978). But in the last instar larvae of *M. brarrricae* and *Spodoptera mauritia* JHA stimulate the prothoracic glands to release β ecdysone (Hiruma *et al.*,1978, Balamani & Nair.,1989a).

JHAs affect the physiology of morphogenesis, reproduction and embryogenesis. Morphogenetic effect is more prominent during larval pupal transformation. Most common morphogenetic effect of JHA treatment is the production of extra larval, nymphal or pupal form (Sehnal.,1971; Novak.,1974; Santha & Nair.,1987). The formation of extra larval instar depends on stage and age of the larvae at the time of treatment. Effects of JH analogue on the morphology of *Aedes scutellaris malayensis collers* (Diptera: Culicidae) was studied. It caused many morphogenetic aberrations on its larvae and pupae (Yodbestra, S. *et al.*, 1985).

Embryogenesis is inhibited if JHAs are applied to eggs. Studies on *Bernisia tabaci* – a sweet potato white fly have revealed a juvenile hormone analogue – Pyriproxyfen is found to suppress embryogenesis and adult formation (Ishaaya I & Horowitz A.R., 1992). Various types of effects ranging from ovicidal effects to delayed effects during post embryonic life have been reported (Riddiford., 1971). Studies on *Pyrrhocoris apterus* - a linden bug have revealed that JHA is effective in preventing the postembryonic metamorphosis of pyrrhocoridae (Linn M. Riddiford and Carroll M. Williams.,1967).

OBJECTIVES OF PRESENT STUDY

3 OBJECTIVES OF PRESENT STUDY

The present study was under taken with the aim of understanding the changes in the developmental stages of *Drosophila melanogaster* after the treatment with a juvenile hormone analogue pyriproxyfen, which is nowadays used as an insect growth regulator for controlling the insect pests. Changes in the developmental stages may possibly indicate its effect on Dipteran pest. Pyriproxyfen is reported to be effective in controlling lepidopteran pest. But its efficacy towards diptera was also documented but to a lesser degree. To study its effect on diptera, a model organism, *Drosophila melanogaster* is selected because of its, short life cycle, ease in rearing and availability of genome sequence. Also we want to study the effects of pyriproxyfen on non targeting organisms.

MATERIALS AND METHODS

4 MATERIALS AND METHODS

4.1 Insect

Drosophila is a genus of small flies, belonging to the family Drosophilidae and order Diptera

4.2 Collection of Eggs and Larvae of *Drosophila melanogaster*

Drosophila stock culture brought from Calicut University cultural lab is maintained in the Zoology laboratory of the college at room temperature.

4.3 Preparation of Culture Media

Drosophila culture media was prepared according to the standard protocol followed by Mysore University. The composition of standard media, used in the present work, is given below.

Rawa	:	100 gms
Jaggery	:	100 gms
Bakers yeast	:	15 gms
Agar agar	:	10 gms
Water	:	1 litre

Plate 1 The life cycle of *Drosophila melanogaster*

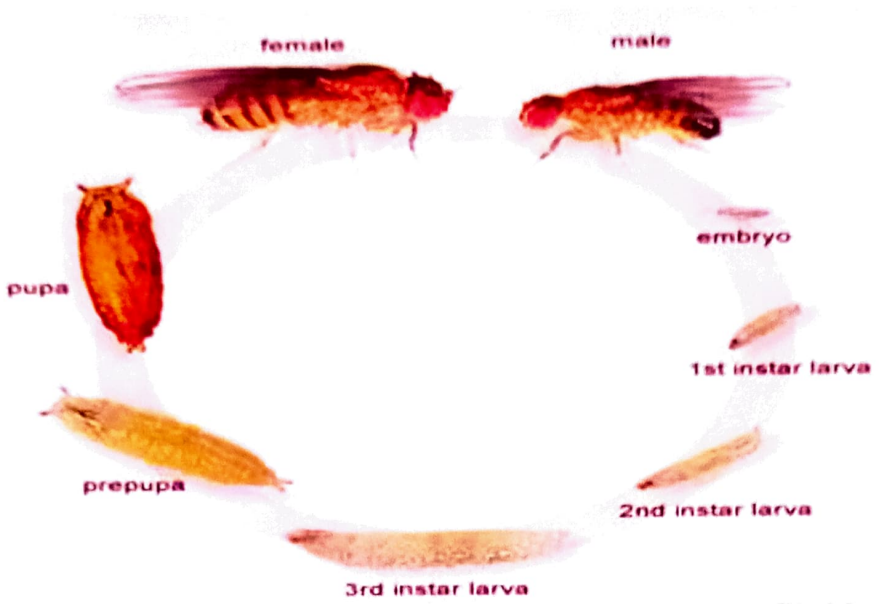


Plate 2 *Drosophila melanogaster* Culture medium preparation

The above medium was boiled in a vessel for 30 minutes and the temperature of the medium was brought to room temperature and 7.5 ml propionic acid was added as a mould inhibitor. The medium prepared was poured into sterile culture bottles. While preparing culture bottles, a uniform quantity of medium by weight was added in all trials (50 ml). Several grains (but not more) of yeast were also scattered to the media surface before adding flies. The bottles were well covered with sterilized cotton clothes.

4.4 Experimental Stages of Insect

1. In order to find the LD_{50} value of Pyriproxyfen toxicity, the third instar larvae of *drosophila* were used. They are larger in size than the 1st and 2nd instar. These larvae were then transferred to the culture media and used for experimental work.

2. For developmental study, adult insects were introduced to each of the test and control culture bottles.

3. In order to calculate the weight of a single larva, 3rd Instar larvae from each of the control and the test bottles were collected in eppendorf tubes and weighed separately.

4.5 Chemical and Treatment

4.5.1 Pyriproxyfen

Pyriproxyfen is a pyridine based pesticide. It is a JH analogue which prevents larvae from developing into adulthood, and thus rendering them unable to reproduce.

4.5.2 Treatments

a) Treatment to test the toxicity of pyriproxyfen.

To calculate the lethal dose, different concentrations of Pyriprixyfen (0.02 µg/µl to 0.08 µg/µl) were prepared and added to the bottle containing 50 ml sterile culture medium. A control bottle was also prepared, using equivalent volume of acetone. The 3rd instar larvae were then transferred to these treated bottle.

Dilution of Pyriproxyfen for Experiment

Pyiproxifen stock- 11.23 %

11.23 % = 11.23 g/100 ml

= 112.3 µg/ µl

Preparation of different dilution of Pyriproxyfen for 50 ml culture bottle

1. Preparation of 0.02 µg/ µl

Stock solution - 112.3 µg/ µl

Take 8.9 µl Pyriproxyfen stock and add 91.1 µl acetone into it and the final solution is added into the 50 ml culture bottle.

2. Preparation of 0.03 µg/ µl

Take 13.35 µl Pyriproxyfen stock and add 86.65 µl acetone into it and the final solution is added into the 50 ml culture bottle.

3. Preparation of_0.04 µg/ µl

Take 17.8µl Pyriproxyfen stock and add 82.2 µl acetone into it and the final solution is added into the 50 ml culture bottle.

4. Preparation of_0.05 µg/ µl

Take 22.25 µl Pyriproxyfen stock and add 77.5 µl acetone into it and the final solution is added into the 50 ml culture bottle.

5. Preparation of_0.06 µg/ µl

Take 26.7 µl Pyriproxyfen stock and add 73.3 µl acetone into it and the final solution is added into the 50 ml culture bottle.

6. Preparation of_0.08 µg/ µl

Take 35.6 µl Pyriproxyfen stock and add 64.4 µl acetone into it and the final solution is added into the 50 ml culture bottle.

Analysis

After a definite time interval, the bottles were checked to count the number of larvae that have been pupated, and compared them with the control larvae.

b) Exposure of Insects to Pyriproxyfen for finding effects on drosophila development.

Sub lethal concentration of pyriproxyfen was selected for the treatment and introduced into the test bottle. A control bottle was also prepared, using equivalent volume of acetone. About 20 female flies and 5 male flies were collected and then introduced into control and test bottle containing 50 ml sterile culture media. Bottles were observed and the developmental stages were noted at regular intervals.

c) Exposure of 3rd Instar larvae to pyriproxyfen for calculating and comparing weight of treated and untreated larvae.

Sub lethal concentration of pyriproxyfen was selected for the treatment and introduced into the test bottle. A control bottle was also prepared, using equivalent volume of acetone. The 3rd instar larvae were then transferred to both control and test bottles. After 12 hours of treatment, the larvae were collected from the control and the test experiment bottles in two eppendorf tubes separately and weighed. The weight of the test and the control larvae were compared.

RESULTS & DISCUSSION

5 RESULTS

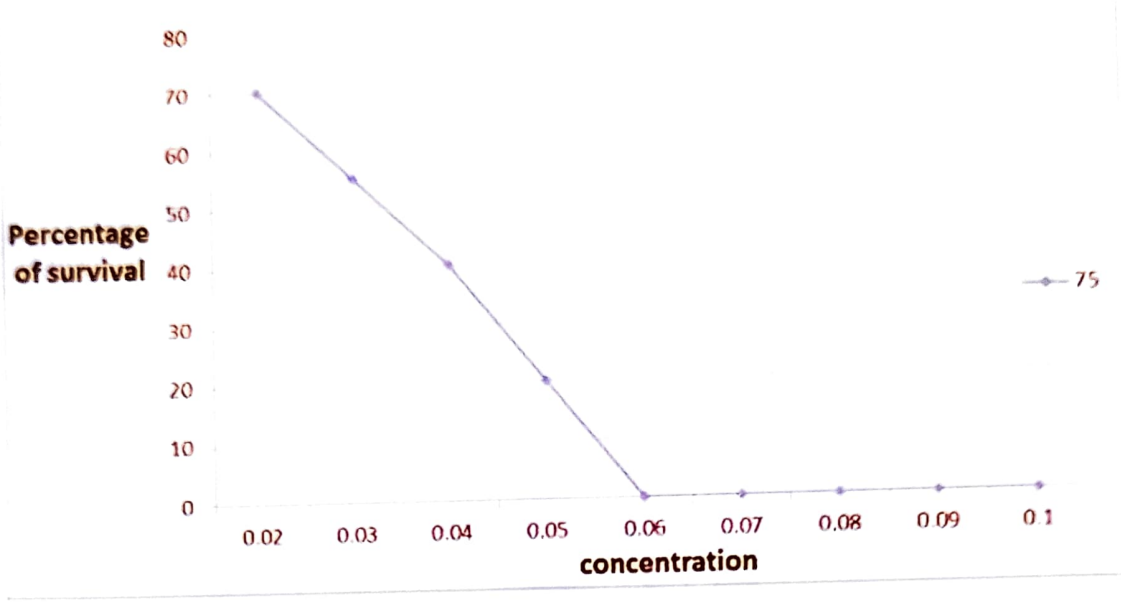
5.1 Toxicity of Pyriproxyfen to 3rd Instar Larvae of *Drosophila melanogaster.*

3rd instar larvae of *D.melanogaster* were exposed to different concentrations (.01 µg/µl,.02 µg/µl,.o4 µg/µl,.06 µg/µl,.08 µg/µl) of Pyriproxyfen, to test the toxicity. Number of larvae pupated after the treatment was counted and compared to that of control. Percentage pupated in the treatment was calculated by taking the number pupated in control as 100%. With increase in the concentration of Pyriproxyfen, the percentage of 3rd Instar larvae survived upto pupation decreased as shown in Table 1.

Table 1 - Toxicity of Pyriproxyfen to the larvae of *D.melanogaster*

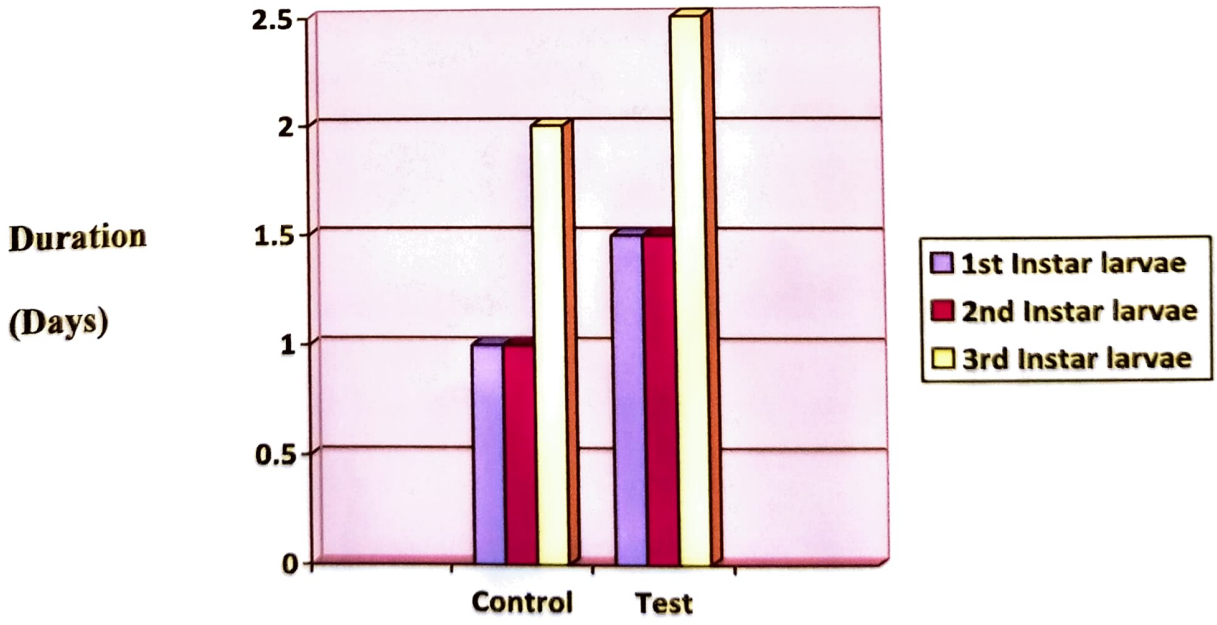
Different concentrations of Pyriproxyfen	Number of LARVAE		Percentage survived in the Test
	Test	Control	
0.08 µg/µl	0	20	0 %
0.06 µg/µl	0	20	0 %
0.05 µg/µl	4	20	20 %
0.04 µg/µl	8	20	40 %
0.03 µg/µl	11	20	55 %
0.02 µg/µl	15	20	75 %

Fig 1. Dose Responsive Curve for LD_{50} Calculation



From the Dose responsive curve that is shown above, the LD 50 value was determined to be 0.035 µg/µl

Fig 2 Graphical representation of the effects of Pyriproxyfen on Drosophila development.



5.2 Exposure of Insects to pyriproxyfen for finding effects on *Drosophila melanogaster* development.

Control with acetone and test with Sub lethal concentration of pyriproxyfen were prepared and flies were added. An analysis was made on the effects perceptible in the test and the control bottles from the 1st instar larvae to the pupae (As a Juvenile hormone analogue, Pyriproxyfen exhibits its maximum efficacy towards larval instars). There was some remarkable changes exhibited in the developmental stages of drosophila melanogaster when they treated with LD 50 concentration of pyiproxifen. Larval stages were extended a little and also exhibited considerable increase in the total period of drosophila lifecycle. Observations are summarized in Table 2

5.3 Exposure of 3rd Instar Larvae to Pyriproxyfen for Calculating and Comparing Weight of Treated and Untreated Larvae.

After 12 hours of treatment with sub lethal concentration of Pyriproxyfen, 28 3rd instar larvae were collected separately from both the control and the test experiment bottles in an eppendorf tube. Both of the eppendorf tubes were separately weighed. The difference between the weight of the test and the control larvae were shown in table 3. From this table, we can clearly identify that there is a significant difference in the weight of the control and the test larvae.

Table 2 Effect of Pyriproxyfen on drosophila development.

Developmental stage	Duration	
	Control	Test
1st Instar larvae	One day (day 1)	One and half day (day 1 to 2)
2nd Instar larvae	One day (day 2)	One and half day (day 2 to 3)
3rd Instar larvae	Two days (day 3 to 4)	Two and half day (day 4 to 6)
Pupae	Five days (day 5 to 9)	Five days (day 7 to 11)

Table -3 Comparison of weight of treated and untreated larvae

Experiment Concentration	Total larvae	Weight of Epitube alone	Weight of Epitube with larvae	Weight of one larva
Control	28	1.01774 g	1.07533 g	2.056 mg
Test	28	1.01284 g	1.06042	1.69 mg

6 DISCUSSION

6.1. Toxicity of Pyriproxyfen to 3rd Instar Larvae of *Drosophila*

Exposure of the 3rd instar larvae of *drosophila* to Pyriproxyfen resulted in the decrease in pupation rate. With the decrease in the concentration of Pyriproxyfen, the percentage of 3rd Instar larvae survived upto pupation increased as shown in Table 1. These effects of Pyriproxyfen were dependent on concentrations, and pupation decreased from 75% (0.08 µg/µl) to 0% (0.02 µg/µl) of control (Table 1).

6.1.1 LD_{50} value

For third instar larvae, the LD_{50} values (as sublethal doses, obtained from a dose response curve) was determined to be 0.035 µg/µl. Above these sub lethal concentrations, more than 50 % of the death of total larvae usually occurred. LD stands for "Lethal Dose". LD_{50} is the amount of a material, given all at once, which causes the death of 50% (one half) of a group of test animals. The LD_{50} is one way to measure the short-term poisoning potential (acute toxicity) of a material. The LD_{50} can be found for any route of entry or administration but dermal (applied to the skin) and oral (given by mouth) administration methods are the most common.

Pyriproxyfen is reported to be effective in controlling Lepidopteran pests. Studies show that, in the last instar larvae of Lepidoptera, JH treatments with in the feeding period causes a delay in metamorphosis for a few days. This has been observed in *Manduca sexta, Mamestra brassicae, Spodoptera littoralis, Laspeyresia pomonella* and *Spodoptera mauritia.* But its efficacy towards diptera was also documented but to a lesser degree. Mostly its effect was studied on Mosquitoes. Effect of Pyriproxyfen were tested against a local population of *Aedes aegypti* (L.) in Iquitos, Peru. Bio assays showed that, when applied to late instars, Pyriproxyfen prevented adult emergence at extremely low concentration (Lc50=0.012ppb). There was no adult emergence from water sampled storage tanks that had been seeded with the equivalent of 50-83ppb of pyriproxyfen.

6.2 Exposure of Insects to Pyriproxyfen for finding Effects on *Drosophila* Development.

There was some remarkable changes exhibited in the developmental stages of drosophila melanogaster when they treated with LD 50 concentration of pyiproxifen. Data represented in table 2 indicates that there is a considerable increase in the duration of larval instars compare to control. Although Pyriproxyfen is reported to be effective mainly on higher instar larval stages of insects, the present study on drosophila melanogaster exhibits its effect on all the three larval instars.

Fruit flies begin their lives as an embryo in an egg. This stage lasts for about one day. During this time embryo develops into a larva. The larval stage is a feeding stage and consists of three subdivisions called instars. After a day, the first instar larva molts and becomes the second instar larva. After a days in this stage, the larva molts again to become the third instar larva. After 2 days of eating in this stage, the larva crawls out of the food source and molts again. Following this molt, the larva stops moving and forms a pupa. Drosophila stays in the pupa for about five days.

Drosophila melanogaster larvae in the control bottle followed the normal developmental pattern and duration. But Pyriproxyfen affected the development of larval instars in test. From the above result, we can clearly understand that the insect growth regulator, Pyriproxyfen can be effectively used against Drosophila melanogaster. It has a significant effect on larval instars of Drosophila melanogaster.

6.3 Exposure of 3rd Instar Larvae to Pyriproxyfen for Calculating and Comparing Weight of Treated and Untreated Larvae.

After 12 hours of treatment with sub lethal concentration of Pyriproxyfen, 28 3rd instar larvae were collected separately from both the control and the test experiment bottles in an eppendorf tube. The difference between the weight of test and the control larvae were shown in table 3. From table 3, we can clearly identify that the weight of the test with sub lethal concentration is smaller

than control. The difference in the weight of the 3rd instar larva may be due to the changes in the protein profile. Larval cell has two types of proteins, membrane proteins and soluble proteins. There may be a considerable decrease in the concentration of either membrane protein/s or soluble protein/s. There is also a possibility for changes in the intensity of protein bands. If we can identify a specific juvenile hormone analogue responsive protein from the protein profile of treated or control larvae, we can target that particular protein for the development of better insect growth regulators with less environmental hazards.

CONCLUSION

7 CONCLUSION

Pyriproxyfen is reported to be effective in controlling Lepidopteran pests. But its efficacy towards Diptera was also documented but to a lesser degree. From the above results we can clearly understand that the insect growth regulator, Pyriproxyfen can be effectively used against Drosophila melanogaster. It has a significant influence on larval instars of Drosophila melanogaster. Although Pyriproxyfen is reported to be active mainly on higher instar larval stages of insects, here the present study on drosophila melanogaster shows its influence on all the three larval instars. With increase in concentration of Pyriproxyfen the percentage of 3rd Instar larvae survived upto pupation decreased. From the present study the LD_{50} value of Pyriproxyfen on Drosophila melanogaster larvae was determined to be 0.035 µg/µl. There was some remarkable changes exhibited in the developmental stages of drosophila melanogaster when they treated with LD_{50} concentration of pyiproxifen. Larval stages were extended a little and also exhibited considerable increase in the total period of drosophila lifecycle. There was also a significant decrease in the weight of the larvae of test experiment compared to control.

The difference in weight of the 3rd instar larva may be due to the changes in the protein profile. There may be a decrease in the concentration of either membrane protein/s or soluble protein/s. There is also possibility for

changes in the intensity of protein bands. If we can identify a specific juvenile hormone analogue responsive protein from the protein profile of treated, we can target that particular protein for the development of better insect growth regulators with less environmental hazards. As a member of Diptera, we can extend the studies on Drosophila melanogaster in to other species of Dipteran pests such as mosquitos and can develop better insect control strategies against these insects.

FUTURE PLAN

8 FUTURE PLAN

In future we can plan experiments to identify and characterize the JH analogue-responsive protein/s and understand its site of synthesis and its regulation by JH analogues in *Drosophila* larvae. As JH is important in the development of insects, identifying the JH-responsive proteins and elucidating their role will be helpful in understanding the mechanism of their action and for designing better insect control strategies. If there is any Pyriproxyfen-responsive proteins identified from the experiments, that can be targeted to develop better insecticides and it will also help in understanding the mechanism of action of IGRSs. *Drosophila*, a member of Diptera, provides a model system for the study and the findings can be extendable to other insects especially members of Diptera including mosquitoes.

REFERENCE

9 REFERENCES

Ackermann M., Bijlsma R., James A. C., Partridge L., Zwaan B. J. and Stearns S. C. 2001 Effects of assay conditions in life-history experiments with *Drosophila melanogaster*. *J. Evol.*Biol. **14**, 199–209.

Adams, T.S. and Filipi, P.A. (1987). Interaction between Juvenile hormone, 20- hydroxy ecdysone, the corpus cardiacum-allatum complex and the ovaries in regulating vitellogenin levels in the housefly, *Musca domestica*. *Journal of Insect Physiology*. 34, 11-19.

Arthur W (1997) The origin of animal body plans: A study in evolutionary developmental biology. Cambridge University Press, Cambridge

Ashburner, M. (1970a). Function and structure of Polytene chromosomes during insect development. Advanced Insect Physiology. 7, 1-95.

Ashburner, M. (1970b). Effect of Juvenile Hormone on adult differentiation of *Drosophila melanogaster*. Nature. 227, 187-189.

Asplen MK, Anfora G, Biondi A, Choi D-S, Chu D et al (2015) Invasion biology of spotted wing *Drosophila* (*Drosophila suzukii*): a global perspective and future priorities. J Pest Sci 88:469–494. doi:10.1007/s10340-015-0681-z

Balamani, E. and Nair, V.S.K. (1989). Prothoracico tropic action of juvenile hormone analogue on neck ligated last instar larvae of *Spodoptera mauritia* Boisd (Lepidoptera: Noctuidae). Entomon. 14, 283-288.

Becher PG, Flick G, Rozpe¸dowska E, Schmidt A, Hagman A, Lebreton S, Larsson MC, Hansson BS, Pis˘kur J, Witzgall P, Bengtsson M (2012) Yeast, not fruit volatiles mediate *Drosophila melanogaster* attr

Benes, H., Edmondson, R.G., Fink, P., Kejzlarovalepesant, J., Lepasant, J.A., Miles, J.P. and Spivey, D.W. (1990). Adult expression of *Drosophila* LSP.2 gen. Dev. Biol. 142, 138-142.

Bownes, M. (1986). Expression of the genes coding for vitellogenin (yolk protein). Annual Review of Entomology. 31, 507-531.

Bownes, M. (1994). The regulation of the yolk protein genes, a family of sex determination genes in *Drosophila melanagaster*. Bio Essays. 16, 745-752.

Chen, P.S. (1971). Biochemical aspects of insect development.

Chen, P.S. and Levenbook. L. (1966). Studies on the hemolymph proteins of the blowfly *Phormia regia* changes in ontogenetic pattern. J. Insect. Physiol. 12, 1595-1609.

Chippindale A. K., Leroi A. M., Kim S. B. and Rose M. R. 1993 Phenotypic plasticity and selection in *Drosophila* lifehistory evolution. I. Nutrition and the cost of reproduction. *J. Evol. Biol.* **6**, 171–193.

Chrysanthis, G., Kaliafas, A.D. and Mintzas, A.C. (1994). Biosynthesis and tissue distribution of four major larval serum proteins during development of *Ceratitis capitata* (Diptera). Insect Biochem. Model. Biol. 24, 811-818.

Comas, D., Piulachs, M.D. and Belles, X. (1999). Fast induction of vitelligenin gene expression by JH III in the cockroach *Blatella germarnica* (Dictyoptera, Blattellidae). *Insect Biochemistry and Molecular Biology*. 29, 821-827.

Daoud, D.S. and Sehnal, F. (1974). Effects of Juvenoids on the Mediterranean fruitfly *(Ceratitis capitata* (Diptera, Tephritidae). Bulletin of Entomological Research. 64, 643-665.

Dhadialla, T.S., Cook, K.E. and Wyatt, G.R. (1987). Vitellogenin mRNA in locust fat body: corrdinate induction of two genes by a JH analog. Development Biology. 123, 108-114.

Dorn, S., Frischknecht, M.L., Martinez, V., Zyrfluh, R. and Fisher, U.Z. (1981). Pflanzen krankh, Pflanzenschutz. 88, 269.

Good T, Tatar M (2001) Age-specific mortality and reproduction response to adult dietary restriction in *Drosophila melanogaster*. J Insect Physiol 47:1467–11473

Hagedorn, H.H. (1994). The Endocrinology of the Adult female mosquito: Advances in Disease Vector Research. 10, 109-148.

Haunerland, N.H. (1996). Insect storage proteins: Genefamilies and receptors. Insect Biochem. Molec. Biol. 26, 755-765.

Henrick, C.A., Stall, G.B. and Siddal, J.B. (1973). Alkyl 3,7,11-trimethyl 2,4-dodecadienoates, a new class of potent Insect growth regulators with JH activity. J. agar. Food Chem. 21, 354-359.

in *Drosophila melanogaster*. *Evolution* **38**, 1004–1010

Ishaaya, I. and Horowitz, A.R. (1992). Novel Phenoxy JHA (Pyriproxyfen) suppress Embryogenesis and Adult emergence of sweet Potato whitefly (Homoptera: Aleyrodedae). Journal of Economic Entomology. 85, 2113-2117.

John, H. Postlethwait (1974). Juvenile hormone and the adult development of *Drosophila*. The Biological Bulletin. 147, 119-135.

Jowett, T. and Postlethwait, J.H. (1981). Hormonal regulation of synthesis of yolk protein and a larval serum protein (LSP_2) in *Drosophila*. Nature. 292, 633-635.

Kaliafas, A.D., Marmaras, V.J. and Christodoula, C. (1984). Immunocytochemical and electrophoretic studies on the localization of the major haemolymph proteins (Ceratitins) in *ceratitis capitata* during development. Roux's Arch. Dev. Biol. 194, 37-43.

Kinjo H, Kunimi Y, Nakai M (2014) Effects of temperature on the reproduction and development of *Drosophila suzukii* (Diptera: Drosophilidae). Appl Entomol Zool 49:297–304. doi:10.1007/s13355-014-0249-z

Lirin Shemshedini and Thomas, G. Wilson (1988). A high affinity high molecular weight juvenile hormone binding protein in the harmolymph of *Drosophila melanogaster*. *Insect Biochemistry*. 18, 681-689.

Lynn, M. Riddiford and Carrol, M. Williams (1967). Effects of JHA on the embryonic development of silkworms. National academy of sciences. 57, 595-601.

Marinotti, D. and de Bianchi, A.G. (1986). Uptake of storage protein by *Musca domestica* fat body. Journal of insect Physiology. 32, 819-825.

Masachika Hirano., Makoto Hatakoshi., Hitoshi Kawada., Yoshiyuki Takimoto (1998). Pyriproxyfen and other juvenile hormones. Reviews in Toxicology. 2, 357-394.

Raff RA (1996) The shape of life: Genes, development, and the evolution of animal form. University of Chicago Press, Chicago

Riddiford, L.M. (1994). Advanced Insect Physiology. 24, 213-274.

Riddiford, L.M. and Law, J.H. (1983). Larval serum proteins of Lepidoptera. In the larval serum proteins of insects: Function, Biosynthesis, Genetic. Ed. Schelter. 75-85. Theme Stuttgart.

Rose M. R. 1984 Laboratory evolution of postponed senescence

Sihuincha, Moises; Zamora-Perea, Elvira; Orellane-rios, Wagner; Stancil, Jeffrey D; Lopez-Sifuentes, Victor; Vidal-Ore, Carlos; Devine, Gregor J. (2005). Potential use of Pyriproxyfen for control of Aedes Aegypti (Diptera: Culicidae) in Iquitos, Peru. Journal of Medical Entomology. 42, 620-630.

Slama, K., Abubakiro, N.K., Gorovitis, M.B., Baltaev, U.A. and Saayov, Z. (1993). Hormonal activity of ecdysteroids from certain Asiatic Plants. Insect Biochem. Molec. Biol. 23, 181-185.

Sliter, T.J., Sedlak, B.J., Baker, F.C. and Schodey, D.A. (1987). Juvenile hormone in *Drosophila melanogaster*: Identification and titer determination during development. *Insect Biochemistry*. 17, 161-165.

Srivastava, U.S. and Lawrence, L. Gilbert (1968). Juvenile hormone: Effects on a higher Dipteran. Science. 161, 61-62.

Steven, J. Kramer., Eric, C. Mundall and John, H. Law. (1980). Purification and properties of Manducin, an aminoacid storage protein of the hemolymph of larval and pupal *Manduca sexta*. Insect Biochemistry. 10, 279-288.

Tatum EL (1939) Nutritional requirements of *Drosophila melanogaster.* Proc Natl Acad Sci USA 25:490–497

Telfer, W.H. and Kunkel, J.G. (1991). The function and evolution of insect storgae hexamers. Annu. Rev. Entomol. 36, 205-228.

Terwilliger, N.B., Dangott, L. and Ryan, M. (1998). Cryptocyanin, Crustacean molting protein: Evolutionary link with arthropod hemocyanins and insect hexamerins. Proc. Natl. Acad. Sci. USA (in press).

Thomas, J. Kelly., Adams, T.S., Mangaret B. Schwartz., Mark J. Birnbaum., Elaine, C. Rubenstein and Richard, B. Imbreski (1987). Juvenile hormone and ovarian maturation in the Diptera: A review of recent results. *Insect Biochemistry*. 17, 1089-1093.

Thomson, J.A., Rodok, K.R., Shaw, D.C., Whitten, M.J., Foster, G.G. and Birt, L.M. (1976). Genetics of Lucilin, a storage protein from the sheep blowfly, *Lucilia caprina* (Calliphoridae). Biochem. Gent. 14, 145-160.

Tochen S, Dalton DT, Wiman N, Hamm C, Shearer PW, Walton VM (2014) Temperature-related development and population parameters for *Drosophila suzukii* (Diptera: Drosophilidae) on cherry and blueberry. Environ Entomol 43:501–510. doi:10.1603/ EN13200

Tu MP, Tatar M (2003) Juvenile diet restriction and the aging and reproduction of adult *Drosophila melanogaster*. Aging Cell 2:327–333

Wheeler, D.E. and Martinez, T. (1995). Storage Proteins in ants (Hymenoptera: Formicidae). Comp. Biochem. Physiol. 112B, 15-19.

Williams, C.M. (1967). Third generation pesticides. Sci. Amer. 217, 13-17.

Wyatt, G.R. and Davey, K.G. (1996). Adv. insect Physiology. 26, 1-155.

Wyatt, G.R. and Pan, M.L. (1978). Insect Plasma Proteins. A rev. biochem. 47, 779-817.

Yodbertra, S., Ketavan, C., Opatham, E.S. and Areckeels (1985). Effects of juvenile hormone analogue on the morphology and biology of *Aedes scutellaris, Malayensis colless* (Diptera: Culicidae) Southeast Asian J. Trop Med. Public Health. 16, 41-80.